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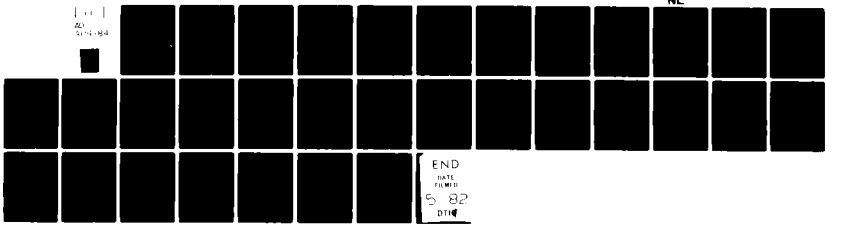

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INSTITUTE REPORT NO. 118

MUTAGENIC ACTIVITY OF N–(n–octyl)–glutarimide AND PROPRIETARY COMPOUND CHR8 USING THE *DROSOPHILA MELANOGASTER* SEX–LINKED RECESSIVE LETHAL TEST

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TOXICOLOGY GROUP,
DIVISION OF RESEARCH SUPPORT

MARCH 1982

Toxicology Series 26

LETTERMAN ARMY INSTITUTE OF RESEARCH
PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129

Toxicology Series 26
--Powers et al

In conducting the research described in this report, the investigation adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care, Institute of Laboratory Animal Resources, National Research Council.

This material has been reviewed by Letterman Army Institute of Research and there is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. *(AR 360-5)*

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Judith M Marshall 12 Nov 92

(Signature and date)

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Drosophila melanogaster, N-(n-octyl)-glutarimide, Proprietary Compound CHR 8

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

N-(n-octyl)-glutarimide and Proprietary Compound CHR 8 are both insect repellents being considered for use among personnel in the field. Both compounds were tested for mutagenic activity by using Drosophila melanogaster Sex-linked Recessive Lethal Assay and were found to be mutagenic after 72-hr feeding exposures to 70 mM and 90 mM of N-(n-octyl)-glutarimide, and 55 mM and 70 mM of Proprietary Compound CHR 8.

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Abstract

N-(*n*-octyl)-glutarimide (CHR 2) and proprietary compound (CHR 8) are both insect repellents being considered for use among personnel in the field. Both compounds were tested for mutagenic activity by using Drosophila melanogaster Sex-linked Recessive Lethal Assay. Both were mutagenic after 72-hour feeding exposures to 70 mM and 90 mM of CHR 2 and 55 mM and 70 mM of of CHR 8.

PREFACE

TYPE REPORT: Drosophila melanogaster Sex Linked Recessive Lethal Assay

TESTING FACILITY: Letterman Army Institute of Research
Presidio of San Francisco, CA 94129

SPONSOR: Letterman Army Institute of Research
Presidio of San Francisco, CA 94129

PROJECT/WORK UNIT/APC: Prevention of Military Disease Hazards, 3M16770A871, Development of Repellents Against Medically Important Arthropods, WU 203, TL09

GLP STUDY NUMBER: 81016

STUDY DIRECTOR: COL John T. Fruin, DVM, PhD, VC, Diplomate of American College of Veterinary Preventative Medicine

CO-PRINCIPAL INVESTIGATORS: CPT Nelson R. Powers, PhD, MSC and CPT Robert A. Wirtz, PhD, MSC

REPORT AND DATA MANAGEMENT: A copy of the final report, study protocol, raw data and SOP will be retained in the LAIR Archives.

TEST SUBSTANCES: N-(n-octyl)-glutarimide (CHR 2)
and
Proprietary compound, (CHR 8)

INCLUSIVE STUDY DATES: 23 February - 1 July 1981

OBJECTIVE: The purpose of this study was to determine the mutagenic potential of CHR 2 and CHR 8 in an invertebrate model.

Acknowledgments

The authors wish to thank Evelyn McGown, PhD, for providing technical assistance and background information concerning formulation of the test substance. The authors also wish to thank SP5 Kincannon, BS, SP5 Alletto, BS, and SP4 Mullen, BS, for their assistance in performing the research.

Signatures of Principal Scientists

Involved in the Study

We the undersigned, believe the GLP Study numbered 81016, described in this report to be scientifically sound and the results and interpretation to be valid. The study was conducted to comply, to the best of our ability, with the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies as outlined by the Food and Drug Administration.

John T. Fruin 12 Mar 82

JOHN T. FRUIN DVM, PhD (DATE)
COL, VC
Study Director

Nelson R. Powers 25 March 82

NELSON R. POWERS, PhD (DATE)
CPT, MSC
Co-Principal Investigator

Robert A. Wirtz 15 Mar 82

ROBERT A. WIRTZ, PhD (DATE)
CPT, MSC
Co-Principal Investigator

DEPARTMENT OF THE ARMY
LETTERMAN ARMY INSTITUTE OF RESEARCH
PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129

REPLY TO
ATTENTION OF:

SGRD-ULZ-QA

4 Feb 82

MEMORANDUM FOR RECORD

SUBJECT: Report of GLP Compliance

I hereby certify that in relation to LAIR GLP study 81016 the following inspections were made:

25 Jun 81/1010 hrs
25 Jun 81/1300 hrs
1 Jul 81/0930 hrs
1 Jul 81/0950 hrs

The report and raw data for this study were audited on 2 Feb 82.

Routine inspections with no adverse findings are reported quarterly, thus these inspections are also included in the 1 Jul 81 and 9 Oct 81 reports to management and the Study Director.

JOHN C. JOHNSON
CPT, MS
Quality Assurance Officer

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The present generation is only a caretaker of the human genome of future generations.

-Malling and Vakovic 1978

Mutagenic Activity of N-(n-octyl)-glutarimide and Proprietary Compound CHR8 using the *Drosophilia melanogaster* Sex-linked Recessive Lethal Test (Toxicology Series 26)--Powers et al

Introduction

To develop better insect repellents for the protection of soldiers from insects and insect-borne diseases in the field is one of the missions assigned to a group of Army researchers. In the last several years, the Division of Cutaneous Hazards, Letterman Army Institute of Research, has tested a large number of chemical compounds submitted by the SRI International, the US Department of Agriculture (USDA) and private industry against a variety of mosquitoes, sand flies, fleas, bugs, ticks and mites in animal and in vitro test systems. Several of these materials have shown sufficient repellent activity and persistence on the skin of animals to warrant consideraton for use in lieu of, or in conjunction with, the current troop-issue insect repellent, 75% N,N-diethyl-m-toluamide (m-DEET) plus isomers in ethanol. The Division of Cutaneous Hazards has also evaluated a number of new formulations of m-DEET prepared at LAIR or submitted by private industry. Several of these new formulations have been more persistent than the current troop-issue repellent in tests on animals.

It is now planned to test the best of the new compounds and formulations on human volunteers to confirm the results that have been obtained in the in vitro and animal tests and to evaluate their performance under conditions of actual use. Before this can be done, it is necessary to obtain certain toxicity data on each compound or formulation to insure that it is safe for application to skin. The toxicity tests required for registration of a new insect repellent are prescribed by the Environmental Protection Agency (EPA). The basic toxicity tests required for experimental use of the new compounds and formulations on human volunteers are prescribed by the LAIR and USAMRDC Human Use Committee. If adverse toxicity data are obtained in these tests, the respective material(s) will be eliminated from consideration, and the prospective tests on human volunteers will not be carried out. The toxicity testing program thereby serves as both a safety factor and secondary screen in the repellent development scheme.

Rationale for SLRL Testing

In addition to the tests for acute and chronic toxicity, evaluation of genetic damage from exposure to chemicals must be considered. A variety of tests using Drosophila are available for the detection of specific types of genetic changes, the most sensitive assay which detects the broadest range of mutations is the sex-linked

recessive lethal (SLRL) test (1-3). This test uses insects of a known genotype and detects lethal mutagenic changes in 800-1000 loci on the X-chromosome, representing 80% of the X-chromosome or 20% of the entire genome (4,5). To date, the SLRL Test has been used in most of the research on the mutagenic response of Drosophila to test substances (1,3,4).

Genetic Basis of SLRL Test

The basic mechanism of the SLRL Test is that the X-chromosome of the father is passed on to the daughter; the sons receive the X-chromosomes from the mother. The recessive lethal genes located on the X-chromosomes are expressed in males in a hemizygous condition, and since the Y-chromosome does not contain the dominant, wild-type alleles to suppress their manifestion, this results in death.

The SLRL Test relies on the fact that among the progeny of females carrying a recessive lethal on one of her X-chromosomes (heterozygous for a recessive lethal), half of the sons will die. By the use of suitable genetic markers, the class of males carrying the X-chromosomes of treated grandfathers can easily be determined. If a lethal was induced, this class will be missing and is easily scored. This test is described as the Basc or Muller-5 test (5,6). The assay system uses strains which prevent the crossing-over in females, heterozygous for the lethal-bearing chromosome; transfer of the lethal from the paternal to the maternal X-chromosome by genetic recombination restores viability of the chromosomes under test and leads to erroneous results, consequently, males receiving the X-chromosome would survive. Since combinations of suitable inversions effectively inhibit the occurrence of crossing over, females used for the test carry two scute inversions; the left-hand part of SC^{S1} and the right-hand part SC^{8} covering the whole X-chromosome, and a smaller inversion In-S in the Basc and dl-49 in the mscy chromosome (5).

Description of Test

The test (7) developed in 1948 for determining genetic changes which in hemiyzgous and homozygous but not heterozygous conditions kill the developing individual (egg to pre adult stage). Such genetic factors, recessive lethals, can be induced on all chromosomes. Only two test generations are needed to detect if sex-linked recessive lethals have been induced on the X-chromosome.

In the SLRL Test, wild-type males, normal round red eyes, are exposed to the test materials (treated). (We use Canton-S (CS)). Such an exposure will be regarded as a recessive lethal if it affects the X-chromosome. These males are mated to homozygous females (we use First Multiple Number 6 (FM6)) carrying the Basc chromosome. This chromosome is expressed as (narrow)-shaped bar eyes, white-apricot in color. The bar serves as a genetic marker in homozygous or hemizygous conditions. It is kidney-shaped in heterozygous females. The progeny of this cross now consists of female heterozygous for the treated X-chromosome, characterized by kidney-shaped red eyes and males of the Basc phenotype that have received their X-chromosome from their Basc mother. Each F_1 female represents one paternal X-chromosome, treated in the male gametes. These siblings are mated to produce the F_2 generation. This generation now consists of males of two phenotypic expressions, those with round red eyes (hemizygous carrying the treated X-chromosome from the F_1 female) and bar-shaped white-apricot eyed males (hemizygous for the Basc chromosome); and females of two phenotypic expressions, kidney-shaped red-eyed (heterozygous, carrying the treated X-chromosome from the F_1 females and the Basc chromosome) and females that are bar-shaped white-apricot-eyed (homozygous for the Basc chromosome). The vials are individually inspected for the presence of males with round red eyes. If this class is missing, it can be concluded that the treated male gamete contained a recessive lethal. Thus this test relies upon the disappearance of a whole Mendelian class (males with round red eyes).

Brooding

As part of the SLRL testing a brooding scheme was used to sample sperm cells exposed to the test substance. This is done as chemicals often exhibit stage-specificity on different stages of germ cell development. The brooding scheme was done at intervals of 3,2,2 and 3 days. This insures that sperm exposed to the test material are in different stages of development: Brood 1 = mature and near-mature sperm; Brood 2 = primarily spermatids; Brood 3 = primarily meiotic stages; and Brood 4 = primarily spermatogonia. This procedure safeguards against the possibility that chemicals with more pronounced effects in earlier stages of spermatogenesis are not erroneously dismissed as false negatives.

Objective of Study

The objective of this phase of the repellent program is to assess the mutagenic potential of two insect repellents, i.e. CHR 2 and Proprietary Compound referred to as CHR 8, by using Drosophila melanogaster in the SLRL Assay.

MATERIALS AND CONDITIONS

Test Substances

CHR 2

Chemical Name: N-(n-octyl)-glutarimide (CHR 2)

CAS: unknown

Molecular structure: $C_{13}H_{13}NO_2$

Molecular weight: 225.3

pH: N/A non aqueous

Physical state: Liquid

Boiling point: 134 F (57 C)

Compound refractory index: unknown

Stability: unknown

Names of contaminants and percentages: *unknown*

Manufacturer: SRI International, 333 Ravenswood Avenue
Menlo Park, California 94025

Manufacturer lot No.: unknown

This sample was kept from exposure light and refrigerated, as required, and thus was not placed in the LAIR Archives.

CHR 8

Chemical name: Proprietary compound, available information on file in LAIR Archives.

A sample of this substance has been placed in the LAIR Archives - GLP 81016

Vehicle: Liposyn™

Chemical name: N/A, mixture

Molecular structure: N/A mixture

Molecular weight: N/A, mixture

pH: 8.0

Physical state: Liquid/white - oil in water emulsion

Boiling point: N/A (*emulsion*)

Compound density: N/A (emulsion)

Contaminants: unknown, refer to Abbott Laboratories, North Chicago, Illinois

Manufacturer lot number: #25-935-DE5

Analytical data: unknown, refer to Abbott Laboratories North Chicago, Illinois

Compound stability: Expiration date, 1 August 1981

Other information: refer to Abbott Laboratories North Chicago, Illinois

Note: This product is approved for use in humans.

Published toxicity data:

Liposyn™ is a commercial preparation of intravenous fat emulsion containing safflower oil, egg phosphaticles, and glycerin.

Both test compounds were water insoluble therefore Liposyn™, a commercial lipid emulsion was used as a vehicle (LAIR SOP-OP-STX-39 water insoluble compounds for SLRL Assay) and was itself appropriate for consumption by the test insects.

Test Model

Insect Genus and Species: Drosophila melanogaster

Strains:

Canton-S (CS), a wild-type stock, characterized by round-red eyes. This stock was selected for *mutagenicity* studies because it has shown a relatively low constant spontaneous mutation frequency (8).

First Multiple Number 6 (FM6) a laboratory stock containing the homozygous Basc chromosome for females and the hemizygous Basc X-chromosome for males. This strain carries the phenotypic markers

for yellow body (Y), bar shaped eye (B) and white-apricot colored eye (W) and several superimposed inversions which prevent "crossing over" (exchange of chromosome segments) with homologous non-inverted X-chromosomes.

Both strains are presently being reared in the insectary at Letterman Army Institute of Research. The original stock colonies were obtained from the University of Wisconsin, Madison, Wisconsin.

Diet The diet is the standard medium used for colony rearing of D. melanogaster; materials and instructions for its preparation are contained in LAIR SOP-OP-STX-5 Drosophila Media Preparation.

Restraint

Ether for anesthesia was used only when conducting matings of F_2 and F_3 generations and for general colony maintenance.

Identification System

Each CS male from the LC_{50}, 72 hour exposure (test, negative, positive control), had a unique number assigned and placed on the vial in which its progeny was produced (LAIR SOP-OP-STX-8 Sex-linked Recessive Lethal (SLRL) Drosophila melanogaster Mutagenicity Test). In this manner progeny were traced back to the parental male which had been subjected to the test compound or controls.

Environmental Conditions

All studies were conducted within the insectary at a temperature of 21 $\pm$ 4 C, relative humidity of 50 $\pm$ 5% and a photoperiod of 12 hours light and 12 hours dark. All insect colonies were reared in polypropylene bottles and SLRL testing was done in glass vials (LAIR SOP-OP-STX-6 Drosophila Stock Colony Maintenance).

Dosing

Dosing was done in compliance with LAIR SOP-OP-STX-7 Drosophila melanogaster Exposure Procedures and LAIR SOP-OP-STX-39 Preparation of Water Insoluble Compounds for SLRL Assay, by allowing the CS strain (wild type male) to feed upon 250 ul of the test chemical formulated with Liposyn and 1% fructose. These males formed the test groups. Concurrent exposure of the Liposyn and 1% fructose (250 ul) for CS males to feed upon were designated as negative control (spontaneous mutation frequency) and 1 mM ethylmethane sulfonate with Liposyn and 1% fructose (250 ul) for CS males to feed upon were designated as

positive control. Ethylmethane sulfonate is a known mutagen and was used to confirm the ability of the test organism to produce SLRL mutagens (9). Dosing was done continuously for 72 hours with test compound replenishment every 24 hours for a total of 3 doses. For test chemical exposure, a pilot toxicity study was done to establish the upper and lower limits of mortality. Based on the results of the pilot study, a second pilot study was conducted. Based on this second pilot study, an exposure level was selected, usually the LC_{50} level for treated males after 72 hours of exposure (4). It was the males from this second pilot study that were used in the SLRL testing. For CHR 2, groups of 100 CS males were exposed to each dose level in the second pilot study of 30, 50, 70, 90 and 110 mM, this was replicated 4 times. For CHR 8, groups of 100 CS males were exposed to dose levels in the second pilot study of 10, 25, 40, 55 and 70 mM again this was replicated 4 times, The male insects surviving the LC_{50} (or as close to the LC_{50} as possible) in each of the 4 replicates were selected for the 4 replicates of the SLRL assay.

Test Format

The CS males surviving the LC_{50} of the test chemical after 72 hours of exposure and those males subjected to the concurrent controls were used in the SLRL assay. Survivors from the test chemical and negative control compound were scored by mating 25 dosed, CS males (wild-type) to FM6-virgin females (Basc chromosome). This was done by placing 3 FM6 virgin females in a vial with one CS male, the vial was labeled with that male's unique number. At intervals of 3,2,2 and 3 days the CS male was transferred to successive groups of 3 FM6 virgin females in vials with that male's unique number. These intervals of days corresponded to broods 1,2,3 and 4. This procedure was replicated 4 times. Scoring of the mutants resulting from positive control exposure was based on mating of 10 CS males using the above mating scheme. This was to be replicated 4 times; however, for the 4th replication, due to limited number only 5 CS males were mated. After sufficient numbers of flies had emerged, a maximum of 25 (minimum of 5) kidney shaped-red eyed F_1 females were selected at random and mated with their brothers (white-apricot bar eyed male). Each pair was placed in an individual vial, and these vials from the same unique numbered father were placed together and labeled with that unique number for reference. After 2 to 3 weeks the F_2 progeny were examined and scored for the absence of round red eyed males, which indicated that a lethal mutation had taken place in the treated male. To confirm this, from each vial scored as a lethal mutation, 3 F_2 females (kidney-shaped red-eyed) were crossed with one bar-shaped white-apricot eyed male. Experimental conclusions were based on the spontaneous mutation frequency (negative control) compared to the mutation frequency induced by the test chemical.

Historical Listing of Significant Study Events

CHR 2

23-27 Feb 81	The formulation of CHR 2 with Liposyn and 1% fructose (LAIR SOP-OP-STX-39 Preparation of Water Insoluble Compounds for SLRL Assay) for pilot toxicity testing to establish the dosing range after 72 hour exposure.
3-4 Mar 81 *	Removal of all adult insects from CS
17-18 Mar 81	colony and collecting of newly emerged
14-15 Apr 81	adult CS males, 24 hours later for toxicity testing (LAIR-SOP-OP-STX-7 Drosophila melanogaster Exposure Procedures).
5-6 May 81	
4-6 Mar 81 *	Selection of CS male survivors
18-20 Mar 81	after toxicity testing and concurrent
15-17 Apr 81	positive and negative control exposure
6-8 May 81	at 72 hours from the concentration that resulted in LC50 (approximately).
6,9,11,13 Mar 81†	Each exposed CS male was crossed with 4 groups of 3 virgin FM6 females in corresponding vials labeled Broods 1,2,3, and 4. Replicate 1.
20,23,25,27 Mar 81†	Replicate 2.
17,20,22,24 Apr 81†	Replicate 3.
8,11,13,15 May 81†	Replicate 4.
23 Mar - 17 Jun 81†	The F2 crosses of all broods for all 4 replicates were made and scored, the F3 confirmation crosses (F3) were made and scored for all 4 replicates.

CHR 8

15-17 Mar 81	The formulation of CHR 8 (LAIR SOP-OP-STX-39 Preparation of Water Insoluble Compounds for SLRL Assay) and pilot toxicity testing as well as all other phases of this test were done in the same manner as with CHR 2.
17-18 Mar 81*	Removal of all adult insects from CS colony
24-25 Mar 81	and collecting of newly emerged CS males
1-2 Apr 81	24 hours later for toxicity testing (LAIR
5-6 May 81	SOP-OP-STX-7 Drosophila melanogaster Exposure Procedures).
18-20 Mar 81*	Selection of CS males from LC50
25-27 Mar 81	(approximately) dose-concentration (LAIR
2-4 Apr 81	SOP-OP-STX-7 Drosophila melanogaster Exposure Procedures).
6-8 May 81	
20,23,25,27 Mar 81†	Each CS male was crossed with 4 groups of virgin FM6 females in vials labeled broods 1 thru 4. Replicate 1.
27,30 Mar 81†	Replicate 2.
1,3 Apr 81†	
4,7,9,11 Apr 81†	Replicate 3.
8,11,13,15 May 81†	Replicate 4.
6 Apr - 1 Jul 81	The F2 and F3 crosses for all broods for all 4 replicates were made and scored.

*dates for each 4 replicates

†dates for each of 4 broods

Statistical Analysis

An adequate sample size was determined by spontaneous mutation frequency. A spontaneous mutation frequency of 0.15% requires the examination of 8,000 to 10,000 X-chromosomes (each culture vial containing F_2 progeny is regarded as an X-chromosome) (10 - 12) We looked at 2,500 X-chromosomes in each of 4 replications in these studies of CHR 2 and CHR 8. The spontaneous mutation frequencies were calculated as 0.043 for CHR 2 and 0.107 for CHR 8; therefore, we were reassured that our sample size was adequate.

Data analysis of mutation frequency of the test compound compared to the spontaneous mutation frequency (negative control) was done by using the Fisher's Exact 2 x 2 table (10,11); a more conservative test, the Kastenbaum-Bowman test (11,12) was also performed. These tests were based on the number of lethal and non-lethal culture vials of the total number examined (each culture vial contains F_2 progeny) from each unique numbered treated male. Also vials without F_2 progeny or less than 5 progeny (F_2) were scored as failure. In addition the mutation frequency of each of the 4 broods was also noted. In one situation (Replicate 4 of CHR 8) clusters (10,11) were observed in the F_2 progeny from a CS male subjected to the test chemical. In such situations where clusters occurred and until statistical treatment of such events are resolved, the investigator as other workers have done, (R. Valencia, PhD, Department of Zoology, University of Wisconsin, Madison, personal communication, 1981), counted the cluster as a single event to avoid biasing the interpretation of results.

RESULTS

Change in Procedure During Study

Chemical analysis was not conducted and composition was assumed to be in accordance with the prescribe formulation. We complied with the following standard operating procedures: SOP-OP-STX-3, Positive Control Substances, SOP-OP-STX-5, *Drosophila* Media Preparation, SOP-OP-STX-6, *Drosophila* Stock Colony Maintenance, SOP-OP-STX-7 *Drosophila melanogaster* Exposure Procedures, SOP-OP-STX-8, Sex-linked Recessive Lethal (SLRL) *Drosophila melanogaster* Mutagenicity Test, SOP-OP-STX-39, Preparation of Water Soluble Compounds for SLRL Assay.

After testing CHR 8 other investigators stated that the compound was supposed to be a clear, odorless, liquid; however, they observed it to be yellow and possess a strong odor. The investigator is unable to determine if the difference in physical properties had an effect on the outcome of the study.

During the study, 7 July 1981, the investigator was informed of improper pesticide use in Letterman Army Institute of Research; however, no detection of such pesticide was noted in the confines of the study area. It is believed that there was no effect upon this study.

The concentrations producing the mean percentage mortality and standard deviation after 72 hours exposure from which surviving males were selected for SLRL testing for each of the 4 replicates of CHR 2 and CHR 8 is shown in Table 1. The 100 CS males concurrently exposed to each negative and positive controls after 4 replications showed a mean mortality of less than 1.0%. The results of the SLRL assay (mutation frequency) for each run and overall results for test and concurrent controls for CHR 2 and CHR 8 are presented in Tables 2 and 3. In addition the analysis of mutation frequency for each brood for test chemical exposure and concurrent controls are presented in Tables 4 and 5 for CHR 2 and CHR 8. All but a few progeny from CS males exposed to concurrent positive controls, 1 mM ethyl methane sulfonate, showed SLRL mutations. Tabular data from this study (GLP #81016) for each male of the test substance CHR 2 and CHR 8 and concurrent negative and positive controls are in the archives of Letterman Army Institute of Research, San Francisco, California.

DISCUSSION

CHR 2

The results of the Fisher's Exact test LAIR SOP-OP-STX-10 Execution of Fortran V Program FEXP, showed significant differences; between the mutation frequency of the negative control and CHR 2; the p value (0.0010) was significant at the 1% level ($p < 0.01$). This indicated a significant difference between mutation frequency (0.208%) due to CHR 2 and the spontaneous mutation frequency (0.043%) due to the negative control. The p value for the extremes was 0.0012. The Kastenbaum-Bowman test revealed significant differences between CHR 2 and the negative control at the 1% level ($p < 0.01$) ($M = 23$, $K = 0.50$).

The analysis of mutation frequency for each brood (Table 4) shows the greatest mutation frequency associated with brood 4. This brood corresponded primarily to the spermatogonia stage (5). Though no statistical analysis was conducted, this indicated that CHR 2 acted upon the spermatogonia of the male sperm. In our study all the CS males fed upon 1 mM ethyl methane sulfonate demonstrated the capability to produce lethal mutations also.

CHR 8

In CHR 8, replicate 4 male T_3-669 fed the compound produced lethal cluster mutations in the F_2 progeny in each of the 4 broods; therefore, each lethal cluster was regarded as representating one lethal mutation. Thus Broods 1,2,3 and 4 instead of having 8,10,9 and 8 lethal mutations were regarded as each having 1 lethal mutation (R. Valencia, PhD, University of Wisconsin, Madison, personal communication, 1981).

The results of the Fisher Exact test LAIR SOP-OP-STX-10 Execution of Fortran V Program FEXP showed significant differences; between the mutation frequency of the negative control and CHR 8; p value (0.0072) was significant at the 1% level ($p < 0.01$). This indicated there was a significant difference between mutation frequency (0.263%) due to CHR 8 and the spontaneous mutation frequency (0.107%) due to the negative control. The p value for the extremes was 0.0114. The Kastenbaum-Bowman test, a conservative test, revealed no significant differences between CHR 8 and the negative control at the 1% level ($p > 0.01$) but found significant differences between CHR 8 and the negative control at the 5% level ($p < 0.05$) ($M = 32$, $K = 0.47$).

The analysis of the mutation frequency for each brood (Table 5) shows an increase in mutation frequency for Broods 2 and 3. These broods corresponded primarily to both spermatids and spermatocytes of spermatogenesis (5). This indicated that CHR 8 acted upon these stages of the male sperm. In addition, one male treated with CHR 8 produced clustering of lethals in all four broods. Such clustering occurred in both the post-meiotic broods indicating spontaneous mutation during development and in pre-meiotic broods which indicated that one mutant cell undergoes division to produce many mutant cells (5). All but a few males in concurrent positive controls fed 1 mM ethyl methane sulfonate demonstrated the capability of this strain to produce lethal mutations.

The recorded mutations (Table 4,5) for insects fed test compounds occurred in the following broods: for CHR 2 Brood 1 = 2, Brood 2 = 5, Brood 3 = 3, Brood 4 = 9; for CHR 8 Brood 1 = 4, Brood 2 = 8, Brood 3 = 7, Brood 4 = 3. Pooling of the brood data into mating A (1-3 days after exposure) and mating B (4-7 days after exposure) resulted in the following: CHR 2 mating A = 2 mutations and mating B = 17 mutations; CHR 8 mating A = 4 mutations and mating B = 18 mutations. The reason for pooling the brood data is that, in mating A, metabolically inactive cells which are easily mutated by direct-acting alkylating or cross-binding agents are tested (13,14). Mating group B test cells which contain active DNA repair systems (4) are able to activate a large variety of indirect mutagens. Based on this concept both CHR 2 and CHR 8 appear to be both direct and indirect mutagens.

CONCLUSION

The mutation frequencies of both substances, CHR 2 and CHR 8, were significantly different from the concurrent spontaneous mutation frequencies suggesting these two chemicals either are, or contain, mutagenic or contain mutagenic contaminants.

RECOMMENDATION

Further investigation about the suitability of statistical tests and further work on identification and statistical treatment of "clusters" should be pursued.

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LIST OF TABLES

Table 1

Concentrations and Corresponding Mean Percentage Mortality for Test Substances Fed to CS Males for SLRL Assay

Replication Number	Conc mM	Mortality* x + sd	Test Substance
1	90	50.00 + 27.00	CHR 2
2	90	79.00 + 23.00	CHR 2
3	90	47.33 + 22.00	CHR 2
4	70	64.60 + 33.50	CHR 2
1	55	50.40 + 24.00	CHR 8
2	70	43.28 + 24.00	CHR 8
3	55	64.36 + 18.00	CHR 8
4	55	52.90 + 27.20	CHR 8

* Based on a sample size ranging from 98 to 101 CS males

Table 2

Sex-Linked Recessive Lethal Assay
of CHR-2*

Compound	Replication 1	2	3	4	Total	% Mutation
CHR-2 †	2/2414	5/2203	4/2137	8/2462	19/9226	0.206
Neg. Cont. ‡	0/2384	2/2216	0/2262	2/2430	4/9292	0.043

* Data are recorded as number of SLRL events/number of X-chromosomes tested.

† CHR-2 in formulation with liposyn and 1% fructose.

‡ Negative control: Liposyn and 1% fructose.

Table 3

Sex-Linked Recessive Lethal Assay
of CHR-8*

Compound	Replication 1	2	3	4 †	Total	% Mutation
CHR-8 ‡	6/2351	4/2159	6/1804	6/2058	22/8372	0.263
Neg. Cont. ∏	2/2216	3/2312	3/2371	2/2430	10/9329	0.107

* Data are recorded as number of SLRL events/number of X-chromosomes tested.

† Clusters - see text

‡ CHR-8 in formulation with Liposyn and 1% fructose

∏ Negative Control - Liposyn + 1% fructose

Table 4

% Lethal Mutation Frequency
for Each Brood of CHR 2*

Compound	Brood 1	2	3	4
CHR 2	0.084	0.214	0.133	0.402
Neg. Cont †	0.000	0.044	0.044	0.087
% Lethal Mutation in CHR 2 ‡	0.084	0.170	0.089	0.315

* CHR 2 in formulation with Liposyn and 1% fructose.

† Negative Control - Liposyn and 1% fructose.

‡ These values are obtained by subtracting the mutation frequency of the negative control from those of the test chemical.

Table 5

% Lethal Mutation Frequency
for Each Brood of CHR 8

Compound	Brood 1	2	3	4
CHR 8*	0.180	0.379	0.326	0.158
Neg. Cont†	0.125	0.131	0.086	0.086
% Lethal Mutation in CHR 8‡	0.055	0.248	0.240	0.072

* CHR 8 in formulation with Liposyn and 1% fructose.

† Negative Control - Liposyn and 1% fructose.

‡ These values are obtained by substracting the mutation frequency of the negative control from those of the test chemical.

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